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Characterization of novel monoclonal antibodies against MERS-coronavirus spike protein

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ABSTRACT

Middle East Respiratory Syndrome coronavirus (MERS-CoV) causes severe pulmonary infection, with ~35 % mortality. Spike glycoprotein (S) of MERS-CoV is a key target for vaccines and therapeutics because S mediates viral entry and membrane-fusion to host cells. Here, four different S subunit proteins, receptor-binding domain (RBD; 358–606 aa), S1 (1–751 aa), S2 (752–1296 aa), and SATM (1–1296 aa), were generated using the baculoviral system and immunized in mice to develop neutralizing antibodies. We developed 77 hybridomas and selected five neutralizing mAbs by immunization with SATM against MERS-CoV EMC/2012 strain S-pseudotyped lentivirus. However, all five monoclonal antibodies (mAb) did not neutralize the pseudotyped V534A mutation. Additionally, one mAb RBD-14F8 did not show neutralizing activity against pseudoviruses with amino acid substitution of L506 F or D509 G (England1 strain, EMC/2012 L506 F, and EMC/2012 D509 G), and RBD-43E4 mAb could not neutralize the pseudotyped I529 T mutation, while three other neutralizing mAbs showed broad neutralizing activity. This implies that the mutation in residue 506–509, 529, and 534 of S is critical to generate neutralization escape variants of MERS-CoV. Interestingly, all five neutralizing mAbs have binding affinity to RBD, although most mAbs generated by RBD did not have neutralizing activity. Additionally, chimeric antibodies of RBD-14F8 and RBD-43E4 with human Fc and light chain showed neutralizing effect against wild type MERS-CoV KOR/KNH/002, similar to the original mouse mAbs. Thus, our mAbs can be utilized for the identification of specific mutations of MERS-CoV.

1. Introduction

Middle East Respiratory Syndrome coronavirus (MERS-CoV) causes an acute and severe respiratory disease with high mortality in humans (van Boheemen et al., 2012). MERS-CoV was first identified in the Kingdom of Saudi Arabia in 2012, which is a single and positive stranded RNA virus (de Groot et al., 2013). As of 8th July 2019, 2,428 laboratory-confirmed cases of MERS worldwide, including 838 associated deaths, with a mortality rate of 34.5 %, were reported. Dromedary camels are widely considered as the source of the transmission of MERS-CoV (Hemida et al., 2017). The rate of human transmission

among household contacts of MERS patients has been approximately 5 % based on serological analysis (Drosten et al., 2014). However, nosocomial super-spreading events occurred in South Korea in 2015 and the rapid and widespread of MERS-CoV from May to July 2015 raised strong concerns regarding the possible generation of mutations with enhanced sequential human infection (Cho et al., 2016).

The spike (S) glycoprotein of MERS-CoV is a critical viral factor for human receptor-mediated infection and is cleaved into a receptor-binding subunit S1 and a membrane-fusion subunit S2 during the infection process (Wang et al., 2013, 2014; Yu et al., 2015). Since the MERS outbreak in South Korea, 13 new viral genomes from 14 infected

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Insect cell codon-optimized gene for S of EMC/2012 strain was synthesized by Bioneer (Daejeon, Korea) and used as a PCR template. Four MERS-CoV spike subunit genes, S_{ATM} (1–1296 aa), S₁ (1–751 aa), S₂ (752–1296 aa), and RBD (358–606 aa), were PCR-amplified with primers containing *Bam*HI site in the forward primer and 6 × His-tag sequence and *Sal*I site in the reverse primer. For S₂ and RBD, the gp67 signal peptide sequence was also included in the forward primer

Table 1
Amino acid sequence of MERS-CoV Spike derived from EMC/2012, England1, and KOR/KNIH/002 strains.

Virus isolate	GenBank Accession No. (Protein ID)	S1	S2
		NTD	RBD
		HR1	TM
		58 91 94 97 137 158 194 289 301 424 431 434 460 482 506 509 510 522 529 530 534 582 833 966 1020 1052 1060 1158 1199 1208 1227 1313 1347	
EMC/2012 AFS88936.1		Y H G P S D H T R T A A S A L F	Q I V V N Q S Q G V A S Q C P
England1 AFY13307.1			H R
KOR/KNIH/ AKL59401.1		R	L
002			

Table 2
Amino acid sequence variation within the receptor-binding domain of MERS-CoV Spike.

Virus isolate		GenBank Accession No. (Protein ID)	Spike RBD												
			424	431	434	460	482	506	509	510	522	529	530	534	582
1	EMC/2012	AFS88936.1	T	A	A	S	A	L	D	D	Q	I	V	V	N
	England1/	AFY13307.1						F							
	England-Qatar	AGG22542.1													
2	Qatar3	AHC74088.1				F									
3	Riyadh1/Bisha1	AGV08379.1/							G						
		AGV08408.1													
4	Riyadh2	AGV08584.1												A	
5	Al-Hasa8c	AGV08438.1													I
6	Riyadh9	AHI48528.1		P			V								
7	Asir2f	AHI48733.1			V										
8	Riyadh345/Riyadh59	AID55095.1/	I												
		AID55090.1													
9	Jeddah_C9313	AID55087.1									H				
10	KOR/CNUH_SNU/122	ALK80291.1								G					
11	KOR/CNUH_SNU/016_06	ALK80192.1										T			
12	KOR/KNIH/002	AKL59401.1											L		

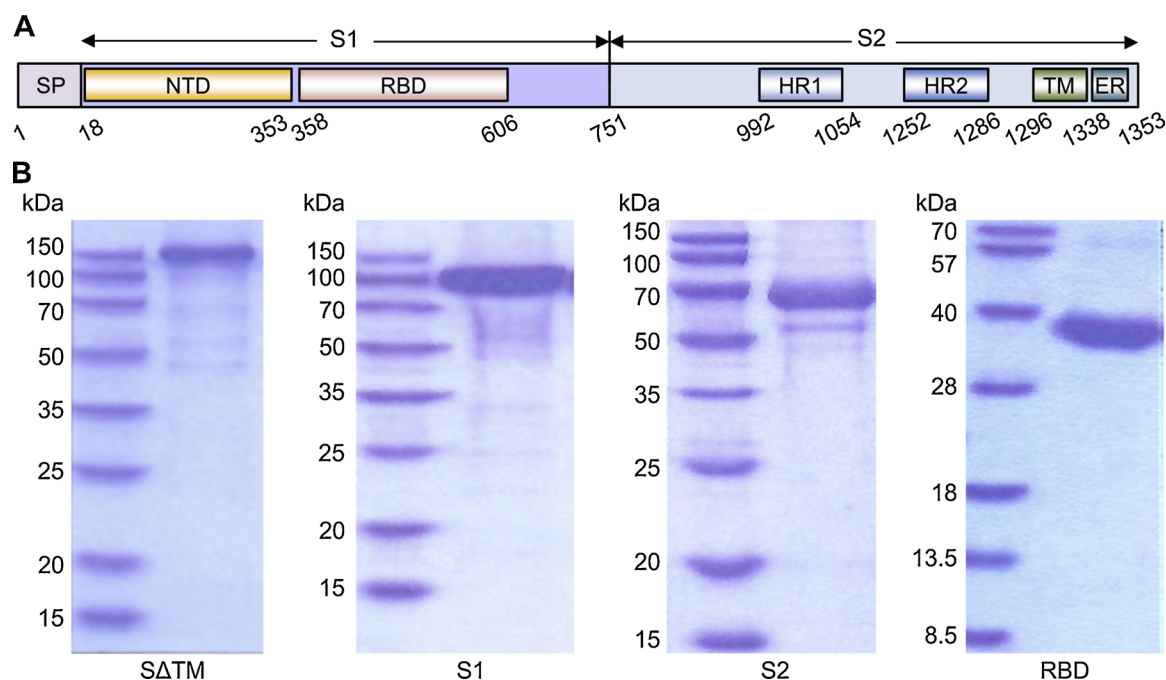


Fig. 1. Production of SATM, S1, S2, and RBD subunit recombinant proteins by baculovirus system. A. Schematic diagram for the domain structure of MERS-CoV Spike (S) protein. B. SDS-PAGE and Coomassie blue staining of purified recombinant S subunit proteins from the insect cell culture supernatant: SATM (1–1296 aa), S1 (1–751 aa), S2 (752–1296 aa), and RBD (358–606 aa).

following the *Bam*HI site. Each PCR product was cloned into the pFastBac donor vector (Bac-to-Bac™ Baculovirus Expression System, Thermo Fisher) using *Bam*HI and *Sal*I restriction sites. Infectious recombinant baculovirus was prepared as described previously (Yang et al., 2015a).

2.4. Production and purification of recombinant S subunit proteins from insect cells

For recombinant protein expression, Sf9 insect cells (2×10^6 cells/ml, 200 ml) were infected with the supernatants containing recombinant baculovirus and cultured for three days (27 °C, 120 rpm), and then the culture media were collected and concentrated by the ammonium sulfate precipitation method (7 M ammonium sulfate). The precipitate was re-dissolved in a buffer solution [20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 5 mM imidazole], and then histidine-tagged proteins

were purified using a metal affinity column with immobilized nickel as a ligand (ADAR Biotech, Rehovot, Israel). The purified protein was eluted from the column with elution buffer [20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 250 mM imidazole], subjected to dialysis in 20 mM Tris-HCl buffer at pH 8.0 containing 10 % glycerol, and then concentrated to 0.25 mg/ml.

2.5. Immunization and hybridoma generation

Female Balb/c mice were purchased from Nara Biotech, Korea. The animal experiments for immunization and collecting ascites were approved by the IACUC (IACUC protocol number ATGen 2016-0113-06 and ATGen 2016-0113-07, respectively). Mice were immunized twice with a 2-week interval with 25 µg MERS-CoV recombinant S subunit protein (SATM, S1, S2, and RBD) combined with 150 µL Freund's complete adjuvant (Sigma, St Louis, CA, USA) via the subcutaneous

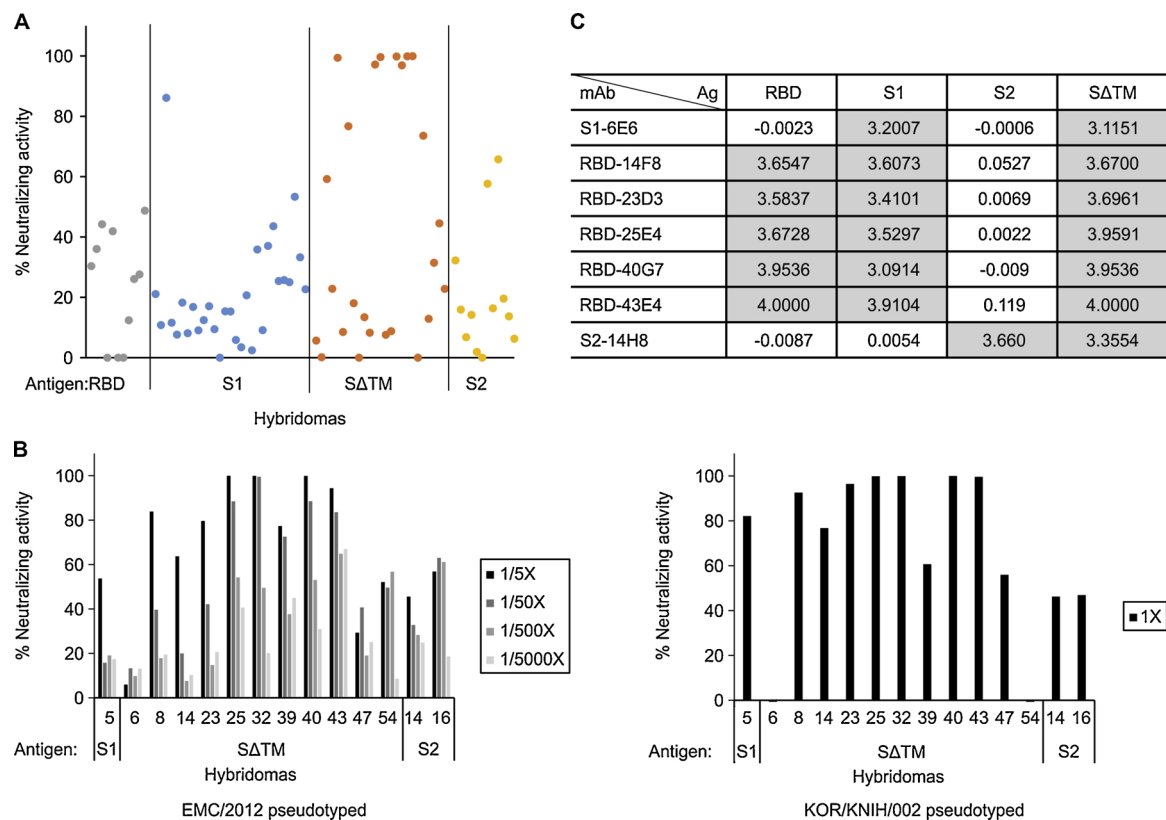


Fig. 2. Pseudovirus neutralizing activity of hybridomas generated using splenocytes of mice that were immunized with SATM, S1, S2, and RBD recombinant protein. A. Distribution of neutralizing activity of 77 hybridomas against MERS-CoV EMC/2012 Spike (S)-pseudotyped lentivirus. Hybridoma culture supernatant was incubated with MERS-CoV EMC/2012 pseudovirions for 60 min at 37 °C and added to 786O cells, incubated for 6 h, replaced with fresh medium, and then analyzed for luciferase-reporter activity 3 days later. The relative luminescence unit (RLU) was converted to % neutralizing activity. B. Neutralizing activity of the selected hybridomas against EMC/2012 (left) and KOR/KNIH/002 (right) strain of pseudovirions. Among 77 hybridomas, selected 15 hybridomas (1 by S1, 11 by SΔTM, and 2 by S2) were tested for their neutralizing activity against indicated pseudovirions. Four ten-fold serial dilutions from 1/5 of hybridoma culture supernatant were used for EMC/2012 pseudovirions, and undiluted culture supernatant was used for KOR/KNIH/002 pseudovirions. C. Binding domain of selected mAbs within S protein. Seven mAbs were purified, and each binding region was assessed using ELISA. The value indicates the OD after coating the 96-well plate with each S subunit antigen.

Table 3
Characterization of mAbs developed by MERS-CoV S subunit proteins.

mAb	Isotype		Specificity	Antigen
	Heavy chain	Light chain		
6E6	IgG1	κ	S1-non RBD	SΔTM
14F8	IgG1	κ	RBD	
23D3	IgG1	λ	RBD	
25E4	IgG1	κ	RBD	
40G7	IgG1	κ	RBD	S2
43E4	IgG2b	κ	RBD	
14H8	IgG2b	κ	S2	

route. The mice were sacrificed 3 or 4 days after the last immunization, and their splenocytes were fused with mouse myeloma cells FO at a 5:1 ratio in the presence of PEG1500 (Roche, Basel, Switzerland). After 10 days of culture with hypoxanthine-aminopterin-thymidine medium, ELISA-positive hybridomas were subjected to limited dilutions and further selected based on pseudovirus neutralization results. For large-scale mAb production, ascites fluid from mice inoculated with the hybridomas was collected and purified using a rProtein A Agarose Resin (Amicogen, Jinju, Korea). Isotype classification of the purified mAbs was performed using the Pierce Rapid ELISA Mouse mAb Isotyping Kit (Thermo) according to the manufacturer's instructions.

2.6. Production of chimeric monoclonal antibodies h14F8 and h43E4

The chimeric mAbs for RBD-14F8 and RBD-43E4 were produced as described previously (Dang et al., 2013). Briefly, RNA was extracted from hybridoma cells and the first-strand cDNA was produced. The V_H and V_L regions were amplified using primers restricted with *SfiI* and *NheI* or *BglII* and cloned into the expression vector to produce chimeric IgG. The sequences of the primers are as follows: 14F8-HC-sfiF, 5'-TTGGTGGCCACAGCGCCGATGTCCACTCGgaggtgagcaggtcggg-3'; 14F8-HC-NheR, 5'-GAGGAGGCTAGCtgcagagacagtgaccagagtc-3'; 14F8-LC-sfiF, 5'-TTGGTGGCCACAGCGCCGATGTCCACTCGgacattgtgatgaccagcttcac-3'; 14F8-LC-BglIIR, 5'-GAGGAGAGATCTatttattccagcttggtcccccctccg-3'; 43E4-HC-sfiF, 5'-TTGGTGGCCACAGCGCCGATGTCCACTCGgaggtgagcaggtgagcaggtcag-3'; 43E4-HC-NheR, 5'-GAGGAGGCTAGCtgcagagacagtgaccagagtc-3'; 43E4-LC-sfiF, 5'-TTGGTGGCCACAGCGCCGATGTCCACTCGgacattgtgatgaccagcttcac-3'; 43E4-LC-BglIIR, 5'-GAGGAGAGATCTtttgattccagcttggtgctccca-3'. Chimeric antibody for RBD-14F8 was named h14F8 possessing human IgG1 Fc and human lambda1 light chain. Chimeric antibody for RBD-43E4 was named h43E4 with human IgG1 Fc and human kappa 1 light chain. Each prepared DNA plasmid was transiently transfected into HEK 293-FT cells (Invitrogen) using polyethyleneimine, and cell culture supernatant (Freestyle 293 Expression media, Thermo Fisher Scientific) was harvested after 7 days. Antibody was purified using Poly-Prep chromatography column (Bio-Rad, Hercules, CA, USA) packed with Protein A. Purified h14F8 (5.37 mg) and h43E4 (2.9 mg) were obtained.

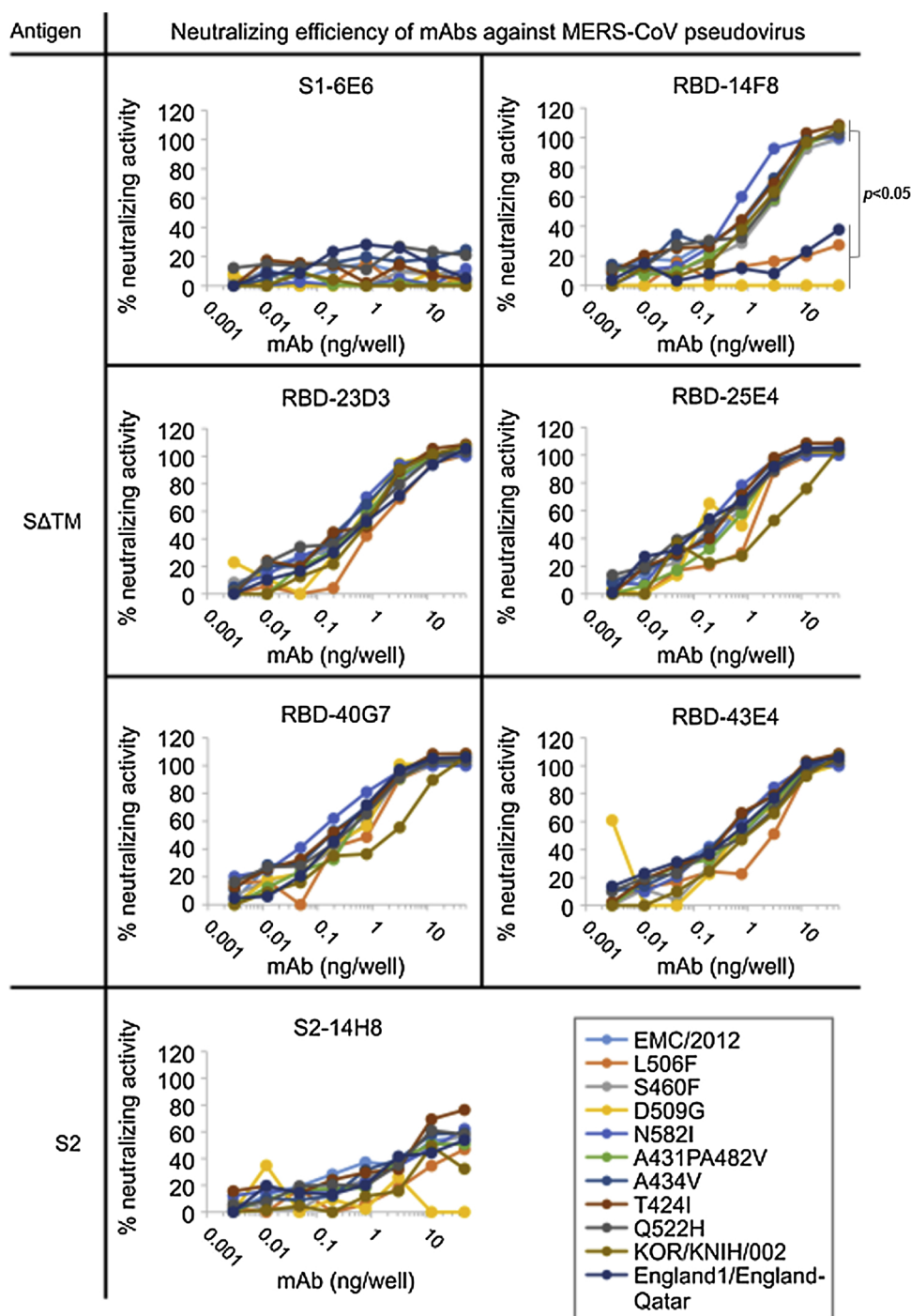


Fig. 3. Dose-dependent neutralizing activity of purified mAbs against 11 different MERS-CoV Spike (S)-pseudotyped virions. Each mAb was diluted 2-fold from 50 ng and tested. mAb was incubated with indicated MERS-CoV S-pseudotyped virions for 60 min at 37 °C and added to 786O cells, incubated for 6 h, replaced with fresh medium, and analyzed for luciferase-reporter activity 3 days later. The RLU value was converted to % neutralizing activity. The data is represented as the mean \pm standard deviation from at least two independent experiments. Statistical significance was determined by one-way ANOVA followed by Tukey's test.

2.7. Pseudovirus production

Lentivirus based MERS-CoV S-pseudotyped particles were generated. HEK 293T/17 cells (4×10^6) were seeded in DMEM without antibiotics into 100-mm Petri dishes at a ratio that will yield 70–90 % confluence at the time of transfection. Lentiviral pseudovirions expressing MERS-CoV S protein were produced by co-transfection of HEK 293 T cells with three plasmids: 7 μ g of pHR'CMV-Luc, 7 μ g of pCMV Δ R8.2 (Naldini et al., 1996), and 100 ng of pCMV/R 8kS encoding plasmids using Lipofectamine 2000 (Invitrogen). The medium was

replaced after overnight incubation. Supernatants containing the viral pseudotyped particles were harvested 48–72 h after transfection, filtered using a 0.45- μ m filter syringe and stored at -80 °C as aliquots (Kim et al., 2007). Titration of pseudovirus was performed using Lentivirus-Associated p24 ELISA Kit (Cell Biolabs, San Diego, CA, USA).

2.8. Pseudovirus neutralization assay

The 786-O cells were plated at a density of 1×10^4 cells/well in 96-well plate with complete DMEM (10 % FBS, 1 % penicillin/

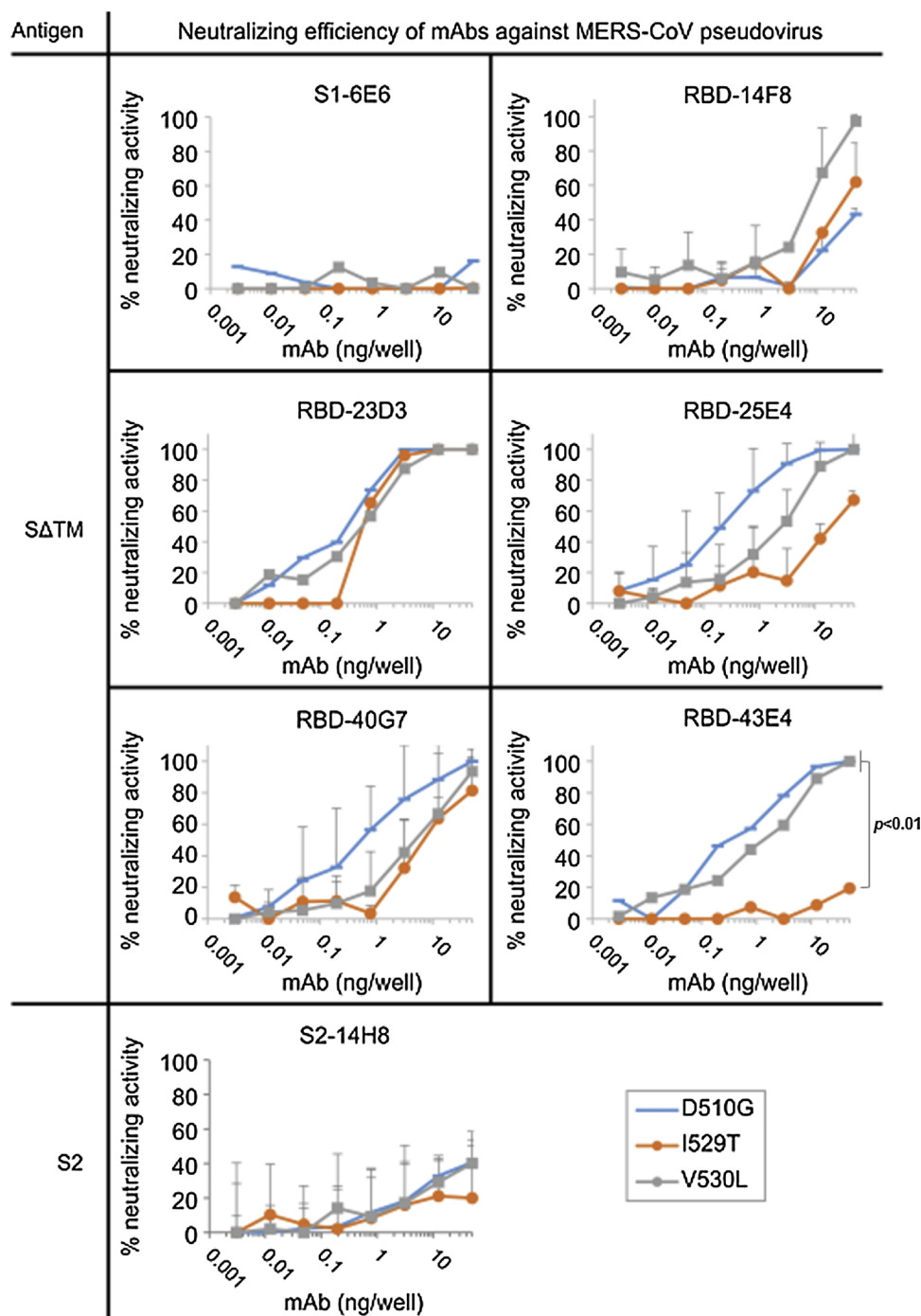


Fig. 4. Dose-response curve of neutralizing activity of mAbs against three pseudoviruses with MERS-CoV RBD identified in 2015 during the Korean MERS outbreak (D510 G, I529 T, and V530 L respectively). Each mAb was diluted 2-fold from 50 ng and tested. mAb was incubated with indicated MERS-CoV S-pseudotyped virions for 60 min at 37 °C and added to 786O cells, incubated for 6 h, replaced with fresh medium, and analyzed for luciferase-reporter activity 3 days later. The RLU value was converted to % neutralizing activity. The data is represented as the mean \pm standard deviation from at least two independent experiments. Statistical significance was determined by one-way ANOVA followed by Tukey's test.

Table 4
Humanized mAb characteristics.

Humanized mAb	Isotype	Light chain
Hu-14F8	hIgG1	hLambda1
Hu-43E4	hIgG1	hKappa1

streptomycin) at 37 °C in a humidified atmosphere of 5 % CO₂ the day before pseudo-viral infection until 95 % confluence was achieved. After incubation of 50 μ L of 2×10^6 MERS-CoV S-pseudotyped particles and 20 μ L of serially diluted mouse immune serum or neutralizing antibody as positive control (Sino Biological Inc., Beijing, China) for 1 h at room temperature, the mixtures were added to the cells and wells were replaced with 100 μ L of fresh media after 6 h of incubation. Cells were

lysed after 72 h with 20 μ L of lysis buffer (Promega, Promega, Madison, WI, USA) and transferred to Opaque plate (Perkin-Elmer, Waltham, MA, USA) (Kim et al., 2007). Luciferase activity was measured by adding 40 μ L of the substrate (Promega) using SpectraMax L Microplate Reader (Molecular Devices, San Jose, CA, USA).

2.9. Plaque reduction neutralization test (PRNT) of wild type MERS-CoV

MERS-CoV (0.004 m.o.i. (multiplicity of infection) in 24 well) KOR/KNIH/002, provided by the Korea Centers for Disease Control and Prevention (Yang et al., 2015b), was pre-incubated with each mAb and then added to Vero cells. After 1 h of incubation, cells were overlaid with a medium containing 1 % Sea-plaque agarose (Lonza, Basel, Switzerland). At 48 h after MERS-CoV infection, cells were fixed and

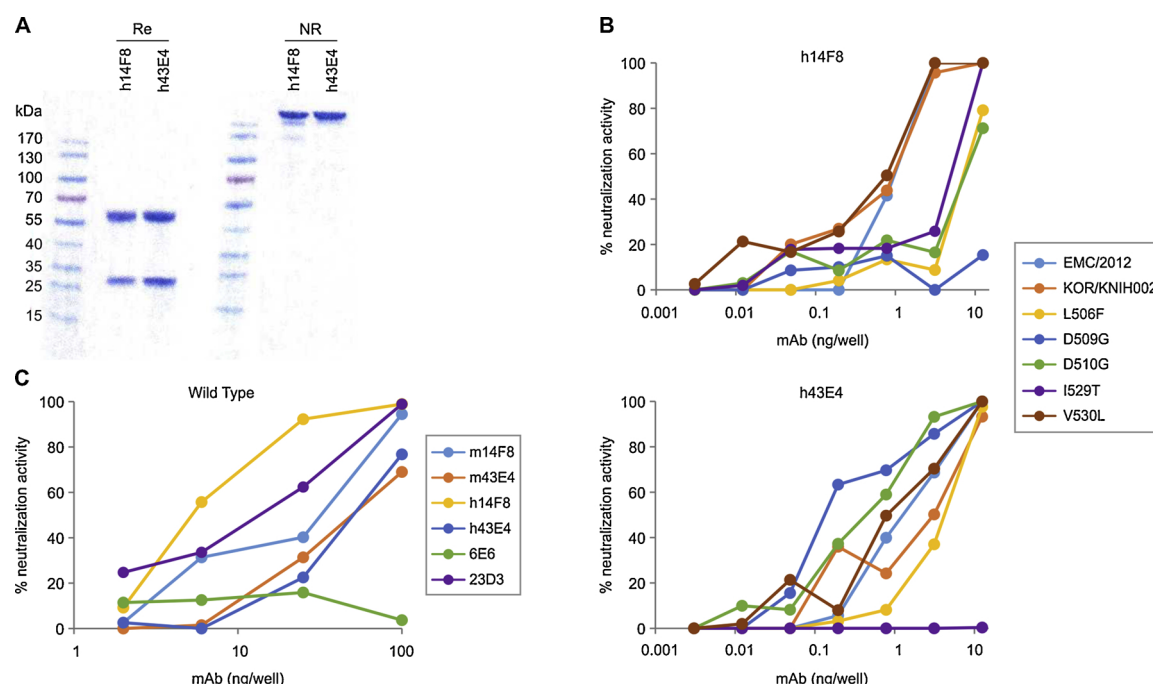


Fig. 5. Epitope-specific neutralizing activity of chimeric h14F8 and h43E4 against MERS-CoV Spike (S)-pseudotyped virions. A. Fc/light chain-humanized chimeric h14F8 (left) and h43E4 (right) mAb purified from 293 F cell culture supernatant after transfection with relevant DNA plasmids and run on the 4–20 % SDS-PAGE followed by Coomassie-blue staining. Re, reducing condition; NR, non-reducing condition. B. h14F8 (left) and h43E4 (right) mAb was diluted 4-fold from 12.5 ng and were neutralized against EMC/2012, KOR/KNH/002, L506 F, D509 G, D510 G, I529 T, and V530 L pseudotyped virions. The luciferase activity of pseudovirus was converted to % neutralization activity. C. Neutralizing activity of both mouse and chimeric 14F8 and 43E4 against wild type MERS-CoV KOR/KNH/002 strain was assessed by PRNT. mAbs were diluted 4-fold from 100 ng and were tested. The data is representative of at least two independent experiments.

stained with anti-MERS-CoV N protein antibody (Sino Biological Inc.) to count the viral plaques.

2.10. Enzyme-linked immunosorbent assay (ELISA)

RBD-, S1-, S2-, and SATM-specific antibody in the hybridoma culture supernatant was measured by ELISA as described previously (Kim et al., 2015; Shere et al., 1997).

2.11. Statistical analysis

Analyses were performed using Prism 5 (GraphPad, San Diego, CA) or Excel program (Microsoft office). Differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test. *P* values of < 0.05 were considered statistically significant.

3. Results

3.1. MERS-CoV S-specific antibody generation from mice immunized with recombinant S subunit proteins

To develop neutralizing mAbs with different epitopes, several S subunit proteins were designed as antigen. The MERS-CoV S glycoprotein consists of a globular S1 domain at the N-terminal region containing the RBD that is responsible for binding to the host cellular receptor DPP4, followed by membrane-proximal S2 domain and a transmembrane domain (Du et al., 2013) (Fig. 1A). Recombinant proteins of SATM (1–1296 aa), S1 (1–751 aa), S2 (752–1296 aa), and RBD (358–606 aa) corresponding to the amino acid sequence of MERS-CoV EMC/2012 strain were produced from the Sf9 insect cells using baculovirus expression system (Fig. 1B). These proteins were immunized into Balb/c mice, and the hybridoma fusion was performed using spleen cells. Then, the culture supernatant of hybridoma cells was subjected to

ELISA to assess whether they secrete the antibody that can bind to S subunit proteins (data not shown). A total of 77 hybridomas secreted antibodies binding to S subunit proteins; 25 from SATM, 29 from S1, 11 from RBD, and 12 mAbs from S2.

3.2. Identification of neutralizing mAbs against MERS-CoV S using pseudovirus system

MERS-CoV S-pseudotyped lentivirus was produced to evaluate the neutralizing activity of antibodies secreted by hybridoma cells. Pseudovirus expressing the MERS-CoV spike protein was generated by co-transfection of the plasmids of HIV-1 Gag/pol, luciferase-expressing HIV-1, and S into HEK 293 T cells. We used S genes without an endoplasmic reticulum signal (*SΔER*) (Kim et al., 2016b). *SΔER* gene of EMC/2012, England 1, and KOR/KNH/002 strains were cloned (Table 1) and the other 13 RBD genes of naturally occurring strains of MERS-CoV were cloned based on the EMC/2012 strain S gene, except for the RBD region (Table 2).

Next, the neutralizing activity of a panel of ELISA-positive 77 hybridomas was evaluated against MERS-CoV EMC/2012 strain S-pseudotyped virions. The inhibition of the pseudovirus infection by antibodies was quantified by luciferase activity in pseudovirus-infected cells (Fig. 2A). Selected clones were further tested against S-pseudotyped KOR/KNH/002 strain (Fig. 2B), and seven clones were selected: six clones (6, 23, 25, 40, 43, and 14) and one clone (14S2) generated by SATM and S2 immunization, respectively. Among these clones, 6 (SATM) did not neutralize both EMC/2012 and KOR/KNH-002 strains, and 14 (S2) inhibited the entry of both pseudotyped strains to the target 786O cells with approximately 50 % activity. All these seven clones were further sub-cloned and finally, mAbs S1-6E6, RBD-14F8, RBD-23D3, RBD-25E4, RBD-40G7, RBD-43E4 by SATM, and S2-14H8 by S2 were purified and characterized for IgG subclass and light chain type (Table 3). Upon examination of the binding domain of the SATM-generated mAbs, S1-6E6 bound to non-RBD S1 and the other neutralizing

mAbs showed affinity to RBD (Fig. 2C). Taken together, several neutralizing mAbs were generated by Δ TM immunization, although the antibodies developed by RBD immunization did not induce a neutralization effect under our experimental condition (Fig. 2A).

3.3. Neutralizing effect of mAbs against various MERS-CoV S pseudoviruses

To evaluate whether the mAbs have cross-protective capability against various MERS-CoV strains, we further examined neutralizing activity against our MERS-CoV S pseudovirus, in which the S gene contained different RBD region of England1/ England-Qatar (L506 F), Qatar3 (S460 F), Riyadh1/Bisha1 (D509 G), Riyadh2 (V534A), Al-Hasa8c (N582I), Riyadh9 (A431 P and A482 V), Asir2f (A434 V), Riyadh345/Riyadh59 (T424I), Jeddah_C9313 (Q522 H), KOR/CNUH_SNU/122 (D510 G), KOR/CNUH_SNU/016_06 (V529 T), or KOR/KNIH/002 (V530 L) strains (Table 2). Of these, the EMC/2012-V534A (Riyadh2) variant exhibited neutralization resistance to our mAbs (data not shown).

Among five mAbs that could bind to RBD, three mAbs (RBD-23D3, RBD-25E4, and RBD-40G7) showed broad neutralizing activity against 14 different S-pseudotyped viruses (Figs. 3 and 4). However, RBD-14F8 mAb did not neutralize three pseudoviruses possessing amino acid substitution of L506 F or D509 G (*S*ΔER of England1 and RBD gene of England1/ England-Qatar and Riyadh1/Bisha1 strain based on EMC/2012 *S*ΔER gene), implying that 506–509 residue mutation of EMC/2012 S can elicit neutralization escape from RBD-14F8 (Fig. 3). RBD-43E4 mAb did not neutralize KOR/CNUH_SNU/016_06 RBD (I529 T) pseudotype (Fig. 4). As expected, S1-6E6 that bound to the non-RBD region of S1 did not show any neutralizing activity even at the highest antibody dose of 20 ng. S2-14H8 mAb, which showed binding affinity to S2, showed partial neutralizing effect compared with other RBD-binding neutralizing mAbs (Figs. 3 and 4).

3.4. Neutralizing effect of humanized RBD-14F8 and RBD-43E4 against pseudovirus and wild type MERS-CoV

To make mAbs available for human clinical use or standard of human sample analysis, RBD-14F8 and RBD-43E4 were genetically engineered to increase their similarity to antibody variants produced naturally in humans; their Fc region was replaced by human IgG1 Fc, and the light chain was replaced with human lambda1 and kappa1 chain (Table 4). The humanized antibody for 14F8 and 43E4 was produced using 293 F cells by transfection of cloned antibody DNA plasmids and purified with Protein A column (Fig. 5A). These chimeric mAbs neutralized the pseudoviruses with S of EMC/2012, KOR/KNIH/002, and V530 L variant of EMC/2012. However, h14F8 showed lower neutralization activity against pseudotyped D509 G, D510 G, and especially L506 F variant, and h43E4 did not neutralize the pseudotyped I529 T variant at all (Fig. 5B), which were also observed in mouse mAb RBD-14F8 and RBD-43E4, respectively.

Neutralizing activity of both mouse and chimeric mAbs of RBD-14F8 and RBD-43E4 were verified by PRNT assay against wild type MERS-CoV KOR/KNIH/002 strain (Fig. 5C). As controls, S2-6E6 and RBD-23D3 were included in PRNT. S1-6E6 did not neutralize, but RBD-23D3 neutralized the wild type strain in a dose-dependent manner, as in previous pseudovirus neutralization. Collectively, chimeric RBD-14F8 and RBD-43E4 mAb showed similar neutralization effect as the original mouse mAb RBD-14F8 and RBD-43E4, respectively, suggesting that these chimeric mAbs can be used to distinguish mutations in specific residue 509 or 529 of MERS-CoV clinical isolates.

4. Discussion

In this study, we aimed to develop mAbs specific to MERS-CoV to utilize them as therapeutics or diagnostic tools of variant MERS-CoV strains. MERS-CoV is a single, positive stranded RNA virus of

approximately 30 kb, which encodes four major viral structural proteins, including S, envelope, membrane, and nucleocapsid as well as several accessory proteins. Since MERS-CoV S mediates viral attachment and fusion to human cells via human cellular receptor DPP4, also known as CD26, we considered S as a target for mAb development including neutralizing antibodies.

We generated mAbs by mouse hybridoma technique using recombinant S subunit proteins as antigens. We selected the 7 mAbs from 77 hybridomas based on the specific binding ability to each S subunit and characterized their neutralizing activity against diverse MERS-CoV S pseudoviruses: six mAbs were developed by Δ TM showing specific binding affinity to non-RBD S1, and RBD; the other one mAb was produced by S2 subunit protein immunization. Polyclonal serum developed by S1 showed high neutralizing activity against both EMC/2012 and KOR/KNIH/002 S pseudovirions, which was comparable to Δ TM immunization (data not shown). However, we failed to obtain potent neutralizing mAbs from S1-immunized mice under our experimental condition. Our RBD-specific mAbs, RBD-14F8, -23D3, -25E4, -40G7, and -43E4, showed higher neutralizing potency than other non-RBD-binding mAbs (S1-6E6 for non-RBD S1, and S2-14H8 for S2), which is consistent with the fact that most reported neutralizing mAbs are RBD-specific (Pascal et al., 2015; Wang et al., 2018; Ying et al., 2014). The mAbs developed by other groups, CDC-C2, m336, and REGN3051, also bind to RBD and potentially neutralize MERS-CoV (de Wit et al., 2018; Pascal et al., 2015; Wang et al., 2018; Ying et al., 2014). Meanwhile, it has also been reported that non-RBD S1-specific mAbs, G2 and 5F9, and S2-specific mAb G4 neutralized MERS-CoV (Chen et al., 2017; Pallesen et al., 2017; Wang et al., 2015; Zhang et al., 2018).

In our study, the RBD subunit protein itself could not induce sufficient neutralizing activity, unlike Δ TM protein, which suggests that the design of antigen to possess an appropriate structural conformation is crucial for developing vaccines and therapeutic neutralizing antibodies. The baculoviral expression system produced all recombinant S subunit proteins in this study. Hence, the baculovirus-expressed Δ TM can be considered as a promising MERS-CoV vaccine antigen given its ability to induce potent neutralizing antibodies, although it does not have trimerization tag for pre-fusion conformation (Pallesen et al., 2017) and may have different glycosylation patterns as compared to mammalian-expressed S proteins (Li et al., 2016).

Our mAbs RBD-23D3, RBD-25E4, and RBD-40G7 targeting RBD showed high cross-neutralizing activities against 14 different MERS-CoV S pseudoviruses. In contrast, the transduction of pseudovirions with the change of V534A in RBD of EMC/2012 was not inhibited by any of our mAbs. Interestingly, two mAbs, RBD-14F8 and RBD-43E4, did not neutralize another pseudovirion with specific mutation in RBD region: RBD-14F8 could not neutralize the pseudovirions with changes in the amino acid residue at 506, 510, and particularly at 509 site (D509 G); meanwhile, RBD-43E4 did not neutralize pseudovirions with the mutation at 529 amino acid residue (I529 T) compared with the EMC/2012 strain S sequence. A single amino acid change in the RBD region was identified in 2015 Korea outbreak patients and it was reported that the mutation of either D510 G or I529 T reduces the viral affinity to human cellular receptor DPP4 (Kim et al., 2016b). In our experiments, lower luciferase activity was also observed in 786O target cells after pseudovirus infection with D510 G or I529 T mutation. The mAb CDC-C2 developed by US NIH possessed binding affinity to RBD and lost neutralizing effect against pseudovirions with mutations at 509, 512, 534, 536, 539, 540, or 542 amino acid residues from EMC/2012 strain S amino acid sequence (Wang et al., 2018). The common amino acid that influences the loss of neutralization between CDC-C2 and our RBD-14F8 mAbs is the 509 residue. S2-binding S2-14H8 showed partial neutralizing activity against all tested pseudoviruses, and non-RBD S1-binding S1-6E6 did not hinder cell entry of the entire tested pseudoviruses at all. More structural data are needed to determine precise epitopes for each neutralizing mAb and the mechanisms

by which combining the mAbs might delay viral escape.

Wild type MERS-CoV KOR/KNIH/002 strain also showed susceptibility to RBD-23D3, -14F8, and -43E4 mAbs, regardless of its origin (either mouse or Fc-/light chain-humanized chimeric antibody). Humanized mAb may be utilized as caliber to measure human immune responses or therapeutic purposes in humans. However, symptoms often appear after the peak of viremia; combined neutralizing mAbs therapy would have to be used before the onset of symptoms to be early enough to avoid viremia. In addition, mAbs targeting non-RBD epitopes of MERS-CoV S may have potential clinical applications in the prevention and/or treatment of disease. With animal models that reflect human MERS-CoV infection, the protective effect of these mAbs may be better assessed.

In conclusion, several mAbs developed by murine hybridoma technique may be utilized as MERS-CoV diagnostics distinguishing S 509 and 529 escape mutant strains or combined therapeutic antibodies with humanized forms.

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CRediT authorship contribution statement

Junghyun Goo: Investigation, Visualization, Writing - original draft. **Yuji Jeong:** Investigation, Validation, Visualization. **Young-Shin Park:** Investigation, Validation, Visualization. **Eunji Yang:** Investigation. **Dae-Im Jung:** Investigation. **Semi Rho:** Investigation. **Uni Park:** Investigation, Visualization. **Hyeyeong Sung:** Investigation, Visualization. **Pil-Gu Park:** Project administration, Investigation. **Jung-ah Choi:** Supervision. **Sang Hwan Seo:** Project administration. **Nam Hyuck Cho:** Resources, Supervision. **Hyeja Lee:** Resources, Supervision. **Jae Myun Lee:** Resources, Supervision. **Jae-Ouk Kim:** Conceptualization, Supervision, Formal analysis, Writing - original draft, Writing - review & editing. **Manki Song:** Funding acquisition, Resources, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare they have no potential conflicts of interest to disclose.

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